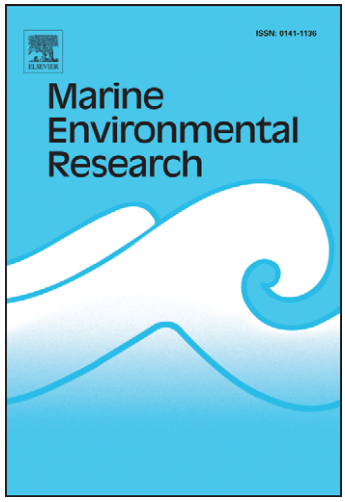
Evidence for estrogenic endocrine disruption in an offshore flatfish, the dab (Limanda limanda L.)

Alexander P. Scott, Matthew Sanders, Grant D. Stentiford, R. Allan Reese, Ioanna Katsiadaki



Evidence for estrogenic endocrine disruption in an offshore flatfish, the dab (Limanda limanda L.)

Alexander P. Scott, Matthew Sanders, Grant D. Stentiford, R. Allan Reese, Ioanna Katsiadaki

PII: S0141-1136(07)00006-2
DOI: 10.1016/j.marenvres.2006.12.013
Reference: MERE 3091

To appear in: *Marine Environmental Research*

Received Date: 8 August 2006
Revised Date: 14 December 2006
Accepted Date: 18 December 2006

Please cite this article as: Scott, A.P., Sanders, M., Stentiford, G.D., Allan Reese, R., Katsiadaki, I., Evidence for estrogenic endocrine disruption in an offshore flatfish, the dab (Limanda limanda L.), *Marine Environmental Research* (2007), doi: 10.1016/j.marenvres.2006.12.013

Evidence for estrogenic endocrine disruption in an offshore flatfish, the dab (*Limanda limanda* L.).

Alexander P. Scott, Matthew Sanders, Grant D. Stentiford, R. Allan Reese and Ioanna Katsiadaki.

Centre for Environment, Fisheries and Aquaculture Science (CEFAS), Barrack Road, Weymouth, Dorset, DT4 8UB, UK

Abstract

Dab (*Limanda limanda*) caught in UK offshore waters show evidence of being exposed to estrogenic endocrine disrupters at a relatively low level. Two of 449 males caught between June and July 2005 had markedly elevated levels of vitellogenin (VTG; 21 and 750 µg/ml) and the remainder ranged from <0.01 to 8.6 µg/ml. Omitting the two outliers, there was a very significant positive relationship with the mass of individual males (a feature noted in previous studies on cod). Mean VTG concentrations in males differed significantly between sites. The site with the highest mean (1.6 µg/ml) was North East of the Dogger Bank and the site with the lowest (0.04 µg/ml) was in Cardigan Bay. Mean VTG concentrations in all North Sea fish were significantly higher than English Channel and Irish Sea fish, but this difference disappeared when fish mass was taken into account. VTG concentrations showed no relationship to water depth, stage of sexual maturity or age of the males. Sixty selected male plasmas were assayed for 17β-estradiol but only two had measurable amounts (assay limit 0.04 ng/ml). Despite being the start of summer, the gonads of many of the males and females (especially those caught in the North Sea) showed signs of sexual maturity (presence of sperm in males and vitellogenic eggs in females). Many females had high VTG concentrations (up to 14 mg/ml) and 78 out of 80 had measurable concentrations of 17β-estradiol. The cause of elevated VTG levels in male dab is unknown. As seen in cod, the presence of affected males does not appear to be linked to proximity to land or to known point sources of endocrine disrupters. However, our data, showing that larger fish are more likely to have elevated VTG concentrations, suggests a gradual accumulation by marine fish,

probably through feeding, of persistent (probably relatively weak) estrogenic compounds.

Keywords: dab; Limanda; cod; endocrine disruption; vitellogenin; VTG; oestrogen; estrogen; marine

Corresponding author. Tel: +44 1305 206637; fax: +44 1305 206601
E-mail address: A.P.Scott@cefas.co.uk (A.P.Scott).

1. Introduction

Contamination of UK freshwaters by estrogenic compounds was first identified through research carried out at the Ministry of Agriculture, Fisheries and Food (now Cefas) and Brunel University in the 1980s. This demonstrated elevated concentrations of the female egg-yolk protein precursor vitellogenin (VTG) in the blood plasma of caged male trout (*Oncorhynchus mykiss*) that were placed in UK rivers – especially near sewage treatment works (Purdom, Hardiman, Bye, Eno, Tyler, & Sumpter, 1994). The finding was extended by Cefas to male flounders (*Platichthys flesus*) in UK estuaries in the 1990s (Allen, Matthiessen, Scott, Haworth, Feist, & Thain, 1999a; Allen, Scott, Matthiessen, Haworth, Thain, & Feist, 1999b) and to cod (*Gadus morhua*) in the open sea within the last five years (Scott, Katsiadaki, Witthames, Hylland, Davies, McIntosh et al., 2006b). Significantly elevated concentrations of VTG were found in plasma of male cod from three areas of the North East Atlantic. Furthermore, the larger the fish, the higher the concentration of VTG that was found. The causative agents are not yet known. However, it is highly unlikely that the main culprits from sewage treatment works (viz. the human female hormones, 17β-estradiol (E_2) and estrone, and the synthetic hormone ethinylestradiol - the main ingredient of 'the contraceptive 'pill') would be able to reach fish so far offshore. Large adult cod are top predators and it is more likely to be due to the gradual accumulation of one (or even a mixture) of the many stable man-made pollutants (some of which are known to be weakly estrogenic) that are widespread in the marine food chain (e.g. DDT, PCB). This hypothesis is

supported by abnormal VTG production being found in males of two other top predators, tuna (*Thunnus thynnus*) and swordfish (*Xiphias gladius*), that inhabit pelagic environments even further from shore (Fossi, Casini, Ancora, Moscatelli, Ausili, & Notarbartolo-di-Sciara, 2001; Fossi, Casini, Marsili, Neri, Mori, Ancora et al., 2002; De Metrio, Corriero, Desantis, Zubani, Cirillo, Deflorio et al., 2003; Fossi, Casini, Marsili, Ancora, Mori, Neri et al., 2004; Desantis, Corriero, Cirillo, Deflorio, Brill, Griffiths et al., 2005). Furthermore, in tuna, it also appears that the larger fish have higher VTG production (Barucca, Canapa, Olmo, & Regoli, 2006).

If, as we hypothesise, the food chain is contaminated with estrogenic endocrine disrupters, we would expect to find males of other marine fish in the waters around the UK with elevated concentrations of VTG in their blood plasma. Indeed, some early work in our laboratory showed that males of a flatfish, the plaice (*Pleuronectes platessa*), caught in the North Sea, all had elevated concentrations of VTG (Scott, Stewart, Allen, & Matthiessen, 2000). However, these fish were caught in the middle of the spawning season and mostly had readily-detectable E_2 in their plasma, so we were unable to conclude whether it was a natural or xenobiotic-induced effect.

As part of an entirely separate study of fish disease in UK waters, scientists at CEFAS Weymouth carry out annual sampling of another flatfish, the dab (*Limanda limanda*), a species that is widely dispersed in the North East Atlantic and, incidentally, a favourite prey item of large cod in the North Sea (Daan, 1973). The samples come from twenty-six stations in the Irish Sea, North Sea and English Channel. The dab is the main offshore sentinel species of the Clean Seas Environmental Monitoring Programme (CSEMP; formerly the National Marine Monitoring Programme), so each sample is accompanied by physical measurements of the site and biometric measurements of the fish.

For the present study, frozen plasma samples were collected from over 1000 male and female dabs during June and July 2005. A further 40 samples of male dab plasma came from a Research Vessel cruise in January, 1997. An antiserum to the egg yolk protein lipovitellin (LV) - which is formed from VTG when it is laid down in the oocytes - plus freshly purified and then freeze-dried dab LV, were used to set up an ELISA. This ELISA was tested for parallelism against purified dab VTG and female plasma samples and then used to quantify VTG in all the male plasma samples

collected in 2005 (n=449) and ca. 270 female plasma samples. E_2 assays were carried out on a sub-set of the plasma samples.

2. Materials and methods

2.1. Purification of dab lipovitellin

Ovaries were removed from reproductively-mature female dabs and stored frozen at – 20 ^{o}C. Prior to extraction, 8 g was placed in liquid nitrogen and ground to a powder in a pestle and mortar. The powder was mixed with 40 ml of ice cold 0.5 M NaCl. After gentle shaking for 30 min, the mixture was centrifuged for 30 min at 1000 G and the supernatant added to 2 l of ice-cold deionised water. The precipitate (crude LV) was allowed to settle overnight at 4 ^{o}C and concentrated by centrifuging. Approximately 1 g of the resultant slurry was mixed first with 1 ml 2 M NaCl and then with 50 ml of Tris-HCL buffer (0.05 M; pH 7.8). Undissolved material was removed first by centrifuging and then by double filtration (0.45 and 0.25 μm pore sizes). The supernatant was loaded on a HiPrep 16/10 QXL ion exchange column (column volume 10 ml; Amersham Biosciences) at a flow rate of 2 ml min^{-1}. Two buffers were made up: A, 0.05 M Tris-HCl pH 7.6; B, 0.05M Tris-HCl pH 7.6 containing 1 M NaCl. The column was equilibrated with 95% A:5% B. The column was developed with a linear gradient from 5% B to 100% B over four column volumes. The effluent was monitored for UV absorption (280 nm) and fractions were collected. There were two major UV peaks (Fig. 1). The fractions corresponding to these peaks were desalted on PD-10 columns with deionised water and freeze-dried. The sharp second peak from the ion-exchange column (B) yielded very little powder (< 5 mg). The other peak (A), however, yielded about 80 mg powder. Both powders were redissolved in carbonate buffer as described for the flounder VTG ELISA (Allen et al., 1999b) and used to coat 96-well polystyrene plates at eight different concentrations (all wells in rows A to H). After washing, the plates were incubated overnight with antiserum to dab LV at 12 different concentrations (all wells in columns 1 to 12). Again, following already-published procedures (Katsiadaki, Scott, Hurst, Matthiessen, & Mayer, 2002), the plates were incubated with 'second antibody' labelled with alkaline phosphatase and developed. Peak B showed no cross-reaction with the antiserum and was discarded. Peak A, however, cross-reacted

strongly with dab LV antiserum. The antiserum was produced in the MAFF, Lowestoft Fisheries Laboratory in 1977 by injection of a rabbit with dab LV purified using previously-published procedures (Plack, Pritchard, & Fraser, 1971).

2.2. Validation of the enzyme-linked immunosorbent assay (ELISA) for dab vitellogenin

Disposables, equipment and basic procedures for ELISA were the same as those used for the assay of stickleback spiggin (*Gasterosteus aculeatus*) (Katsiadaki, Scott, Hurst, Matthiessen, & Mayer, 2002). For coating plates, 1 mg heat-treated LV was dissolved in 500 µl distilled water followed by 500 µl of carbonate buffer. A 400 µl aliquot was added to 100 ml of coating buffer and 100 µl of this dilute solution was then added to all wells. The antiserum was used at a final dilution of 1:42,000 (v/v). Tests were carried out for repeatability of observations between plates (within an assay); repeatability between assays; parallelism between standard LV, standard VTG and diluted plasma samples; and comparability between plasma samples assayed first with LV as the plate-coating material and then VTG as the plate-coating material.

2.3. Collection of dab

Starting on 17 June 2005, male and female dab were captured from twenty-six sites in the Irish Sea, English Channel and North Sea using 30 min tows of a standard Granton Trawl. Sex, size (total length and weight) and external signs of disease were recorded. Blood samples were taken from the caudal vein using heparinised syringes, centrifuged for 10 min and the plasma snap frozen in liquid nitrogen and stored – 20 ^{o}C. The fish were sacrificed by a blow to the head, followed immediately by severing of the spinal cord. The otolith was removed for age determination. Gonads (females only) and livers were weighed in order to calculate the Gonadosomatic Index (GSI) and Hepatosomatic Index (HSI), respectively (= 100 x [organ weight/bodyweight]). Samples of liver, kidney, spleen and gonad were fixed in neutral-buffered formalin for 24 h followed by transfer to industrial methylated spirit. Fixed samples were processed to wax in a vacuum-infiltration processor using standard protocols. Sections were cut at 3 to 5 µm on a rotary microtome and mounted onto glass slides before staining with haematoxylin and eosin. Stained sections of the gonads were examined

by light microscopy to a) check the original visual analysis of sex and b) determine the stage of reproductive maturity of the fish. Testes that showed no signs of sexual maturation were given a score of 1; and those with sperm or spermatocytes present were given a score of 2. Ovaries with only primary oocytes or oogonia present (immature) were scored as 1; those with cortical alveoli stage oocytes as 2; those with oocytes undergoing secondary vitellogenesis (VTG incorporation) as 3; and those with atretic yolky oocytes as 4. Mean scores for each sex at each site gave maturity indices. About 10 fish showed discrepancies between sex as recorded initially and as determined from histology. These fish were omitted from statistical analyses.

2.4. Statistical analysis

Data were analysed using Stata version 9.2 (2006 StataCorp, College Station, TX, USA). After scanning using a variety of exploratory data analysis techniques, log(VTG) in males was modelled as a response variable with discrete and continuous predictors with and without the two highest values. Logs were used to make the empirical distribution symmetrical. VTG values below the level of quantification were allocated a censored value of 0.01 µg/ml, prompting the use of Tobit regression to obtain unbiased estimators of left-censored data. The model was simplified by excluding or grouping factor values whose effects were not significant (based on t-tests of ML estimators). An emphasis was put on producing a biologically meaningful interpretation rather than an arbitrary mathematically "best" model.

3. Results

3.1. Validation of the ELISA

Plasma samples from four female dabs gave dilution curves that were parallel to both LV and VTG over the steepest, most accurate, part of the curve (Fig. 2). In all assays, a pool of female plasma was included in every plate. Three assays were carried out. The first assay yielded a within-assay (essentially between-plate) estimate (mean ± SE) for the plasma pool of 36.1 ± 2.4 µg/ml (n= 11). The second assay gave an estimate of 38.9 ± 0.9 (n= 3) and the third 35.72 ± 4.2 (n= 8). A pool of male plasma in the first assay gave an estimate of 4.8 ± 0.2, n= 11. All male plasma samples were

assayed on the same date (with a single batch of standard and reagents) in order to minimise inter-assay variation.

3.2. VTG in males

Weight was strongly related to length for males; over the limited size range, linear and cubic functions gave identical goodness of fit ($r^2 = 0.86$). Slopes were similar, but intercepts were significantly different in each sea area. Males in the North Sea were on average larger than those in the other two sea areas. The mean ± SE values were: North Sea, length 21.3 ± 0.1, weight 96.8 ± 1.8, n = 182; Irish Sea, length 19.5 ± 0.2, weight 76.8 ± 2.0, n = 182; English Channel, length 20.0 ± 0.2, weight 90.0 ± 2.4, n = 85.

Mean male VTG concentrations for each site are shown in map form in Fig. 3. Locations of the sites are listed in Table 1 and summary statistics in Table 2. Two males with VTG concentrations higher than 10 µg/ml were treated as outliers. These data are shown separately (Table 3). Of the 1120 fish collected during 2005, 449 were male. The distribution of VTG concentrations in these males was: <0.01 µg/ml, n = 50; 0.02 to 0.1 µg/ml, n = 132; 0.1 to 1 µg/ml, n = 222; 1 to 10 µg/ml, n = 43; >10 µg/ml, n = 2.

Mean VTG concentrations in male dab in the North Sea (0.53 ± 0.08, n = 181) were marginally, but nevertheless significantly, higher than those in the Irish Sea (0.34 ± 0.07, n = 181) and English Channel (0.25 ± 0.05, n = 85). With fish weight as a covariate, the differences disappeared. In essence, the difference between the seas was driven by log(VTG) being positively related to fish weight: all fish (Fig. 4), r = 0.290, n = 447, p<0.0001; North Sea, r = 0.204, n = 182, *p*<0.005; Irish Sea, r = 0.358, n = 182, *p*<0.0001); English Channel, not significant.

Mean VTG concentrations were clearly very different between the twenty-six sites (Fig. 3). Statistically, with or without fish weight as a variate, there were at least eight separate overlapping groupings of sites by post-hoc statistical analysis. However, sites with very low mean VTG concentrations and those with mean VTG concentrations that were up to 30 times higher were scattered across the sea areas. Nevertheless, the map shows clusters of sites within which the values were very similar to each other (e.g. Cardigan Bay, the eastern English Channel and Liverpool Bay).

Based on summary statistics (Table 2), there was no relationship between mean VTG concentrations and water depth in males.

The mean concentration of VTG in fish with completely immature testes (0.47 ± 0.14, n = 164; stage 1) did not differ significantly from that in fish with sperm in their testes (0.43 ± 0.04, n = 283; stage 2). Mean male VTG concentrations at the different sites also showed no relationship to the stage of maturity of the females or to the date of capture. The forty male dab collected from the North Sea at the end of January 1997 had a mean length of 19 cm, a mean weight of 74 g and a mean VTG concentration of 0.62 ± 0.07 µg/ml (not dissimilar to 0.53 ± 0.08 for North Sea males in July, 2005).

There was a significant positive relationship between male log(VTG) and HSI ($r = 0.276$, $n = 357$, $p<0.0001$). Mean HSI values at some sites were twice as high as those at other sites (Table 2), but this was not related to mean VTG levels. There was a very significant positive correlation between male and female mean HSIs (results of analysis not shown). This suggests that whatever causes between-site differences in HSI, it affects both sexes equally.

Fifteen fish in each of the following categories were chosen for E_2 assay: immature testes and mean VTG = 0.08 µg/ml; immature testes and mean VTG = 3.3 µg/ml; mature testes and mean VTG = 0.05 µg/ml; mature testes and VTG = 2.7 µg/ml. E_2 could not be detected (<0.04 ng/ml) in any but two males. One of these males, with a VTG concentration of 5 µg/ml had an E_2 concentration of 1 ng/ml. The other male, with <0.01 µg/ml VTG, had an E_2 concentrations of 0.13 ng/ml.

Age data (from otoliths) were available only for males caught on the Dogger Bank (sites 18 to 21). Some of the males were as much as 12 years old. However, there was no relationship between log(VTG) concentrations and age (Fig. 5). The positive relationship of log(VTG) to fish size, on the other hand, was particularly high ($r = 0.468$, $n = 88$, $p<0.0001$) for this group of fish (Fig. 6).

3.3. VTG in Females

The first ten females caught at each site were analysed for VTG. There were highly significant differences in mean VTG concentrations in females between the North Sea (2091.2 ± 219.8, n =104), the Irish Sea (409.4 ± 56.1, n = 107) and the

English Channel (190.8 ± 43.2, n = 58). The site differences are very obvious in the summary statistics (Table 4).

As with males, there was a significant positive relationship between log(VTG) concentrations and fish weight (r = 0.241, n = 269, p<0.0001).

Females with ovaries that contained atretic eggs (stage 4; 2452.5 ± 433.3, n = 38) or oocytes in the early stages of vitellogenesis (stage 3; 2578.6 ± 415.6, n = 30) had significantly higher VTG concentrations than those with ovaries that contained primary (stage 1; 424.0 ± 76.3, n = 125) or cortical alveoli-stage (642.2 ± 91.6, n = 76) oocytes only. A positive relationship was evident between log(VTG) concentrations and log(GSI) (r = 0.583, n = 235, p<0.0001). There was also a significant correlation between mean VTG concentrations and the mean female maturity index at all sites (r = 0.70, n = 26, p<0.0001). Mean maturity index of females in the North Sea (2.68 ± 0.10, n = 104) was significantly higher than that in the Irish Sea (1.19 ± 0.05. n = 107) and English Channel (1.95 ± 0.13, n = 58). Considering that females in the North Sea had higher mean VTG concentrations than those in the other two sea areas and that sampling was spread over 21 days starting in the Irish Sea and ending in the North Sea, it was not surprising that a significant relationship could also be found between log(VTG) concentrations and date of capture (r = 0.291, n = 269, p<0.0001). Unlike the males, there was no significant correlation between log(VTG) concentrations and HSI in females.

Twenty plasma samples were selected for E_2 assay from each of the four stages of maturity of females. Within each stage, ten samples were taken from the bottom of the distribution of VTG concentrations and ten from the top. There were no significant differences in E_2 concentrations between the different groups, apart from those females in which oocytes were undergoing vitellogenesis and had high VTG concentrations (Fig. 7). This is consistent with what is known about the major role of E_2 in females (i.e. stimulating vitellogenesis in the liver). Only two (out of 80) females had E_2 concentrations below the limit of the assay. One fish at the primary oocyte stage and with only 5 µg/ml VTG had an apparently very large E_2 concentration of > 20 ng/ml. This was omitted from the statistical comparison between VTG and E_2 concentrations.

Age analysis was only carried out for females caught on the Dogger Bank (sites 18 to 21). The oldest female was 8 years old. VTG was not related to either age or water depth in females.

4. Discussion

This study set out to determine whether dab caught in offshore waters of the UK show any evidence of exposure to estrogenic endocrine disrupters. The answer is in the affirmative. Of the 449 males caught in June/July 2005, one had a markedly elevated (750 µg/ml) and another a moderately elevated (21 µg/ml) concentration of VTG and the remainder ranged from <0.02 to 8.6 µg/ml. Without the two outliers (and even including the lower one), there was a very significant positive relationship with the size of the males. This relationship has already been noted in previous studies on cod and tuna.

Mean VTG concentrations differed between sites, but there was no clear pattern. Sites that might have been expected to have a large proportion of affected males - for example, close to the Tees estuary, the sediments of which are known to be contaminated with estrogenic compounds (Thomas, Balaam, Hurst, Nedyalkova, & Mekenyan, 2004) - showed no evidence of contamination at all.

As in male cod (Scott et al., 2006b) and male flounders (Scott, Katsiadaki, Kirby, & Thain, 2006a), there was no evidence that elevated VTG concentrations in male dabs were related to their stage of reproductive maturity or to plasma E_2 concentrations.

4.1. Comparison with previous studies on estrogenic EDs on marine fish

In addition to the findings on cod and dabs, there is now a large body of evidence for the existence of estrogenic endocrine disruption in the marine environment. Male flounders (*Platichthys flesus*) caught in industrialised estuaries of the UK and the Netherlands have been found with elevated concentrations of VTG in their plasma (Lye, Frid, Gill, & McCormick, 1997; Lye, Frid, & Gill, 1998; Allen et al., 1999a; Allen et al., 1999b; Matthiessen, Allen, Bamber, Craft, Hurst, Hutchinson et al., 2002; Vethaak, Lahr, Kuiper, Grinwis, Rankouhi, Giesy et al., 2002; Kirby, Allen, Dyer, Feist, Katsiadaki, Matthiessen et al., 2004; Kleinkauf, Scott, Stewart, Simpson, & Leah, 2004b; Kirby, Smith, Barry, Katsiadaki, Lyons, & Scott, 2006). The range of VTG concentrations was great (from <0.01 µg/ml to >50 x 10^3 µg/ml). Some male flounders with elevated VTG concentrations have also been

caught in the open sea (Allen et al., 1999a), but these were hypothesised to be fish that had recently emigrated from a contaminated estuary. In estuarine and coastal areas of the USA (Mills, Gutjahr-Gobell, Horowitz, Denslow, Chow, & Zaroogian, 2003; Roy, Armstrong, Sakamoto, Steinert, Perkins, Lomax et al., 2003) and Japan (Hashimoto, Bessho, Hara, Nakamura, Iguchi, & Fujita, 2000; Hara, Matsubara, & Soyano, 2001; Ohkubo, Mochida, Adachi, Hara, Hotta, Nakamura et al., 2003), many male fish have also been found with high concentrations of VTG in their plasma.

In all the above studies, fish were caught either just offshore or in estuaries with a known high level of contamination. In these situations, it is difficult to dismiss the effects of sewage treatment works, fish farming and nearby industries. Evidence of estrogenic effects 'in the open sea' that can be directly compared to that in cod (and dab in the present study), comes from studies on swordfish and tuna. VTG production has been demonstrated in male swordfish from the Mediterranean and from the waters around South Africa (Desantis et al., 2005). Similar to the findings in dab and cod, there also appears to be a positive relationship between VTG levels and fish size in male tuna (Barucca et al., 2006).

4.2. What is the most likely route of exposure?

If the causative agents were absorbed directly from the water, then one would expect, firstly, that all fish would be affected equally regardless of size and, secondly, that there would not be marked differences in mean VTG concentrations between sites within a sea area. Both arguments are admittedly weak as fish may get more sensitive to estrogens as they grow and currents and tidal flows also ensure that sea water is not homogeneous. More convincing is the fact that, in cod, dab and tuna, VTG concentrations are positively related to the size of the fish. The most important determinant of size is the amount of food that a fish has eaten, so it seems plausible that the main route of entry is via the food. This is consistent with VTG concentrations in dab being typically 10 to 20 times lower than those we found in cod. Large cod (the ones in which one is most likely to find elevated VTG concentrations) are top predators (i.e. they prey on other fish, including dabs) and are thus one level up the food chain.. It has been well documented that biomagnification of lipophilic pollutants in aquatic food chains is positively correlated to both trophic level

(Burreau, Zebuhr, Broman, & Ishaq, 2006) and fish size (Olsson, Valters, & Burreau, 2000).

There are few endocrine disrupter studies in the freshwater literature to support our hypothesis. This is because, although cumulative and persistent weak estrogenic chemicals are almost certainly present in the freshwater environment, their effect would be very difficult to demonstrate in an environment dominated by short-lived but nevertheless far more potent compounds emanating from sewage treatment works and factories. However, one study (Jobling, William, Johnson, Taylor, Gross-Sorokin, Nolan et al., 2006) shows a strong positive relationship between the frequency of intersex and the age of roach (*Rutilus rutilus*) living in estrogen-contaminated UK rivers (i.e. there is evidence of a cumulative estrogenic effect). Another study (Vine, Shears, van Aerle, Tyler, & Sumpter, 2005), that looked for effects in a top predator, the pike (*Esox lucius*) preying on estrogen-contaminated roach, found that, although the bile of male pike caught downstream had significantly more estrogenic activity than that of male pike caught upstream, there was no significant difference in VTG concentrations. Only two out of fifty-two male pike had detectable VTG in their plasma. Unfortunately, fish size was not taken into account in that study.

4.3. What is the likely identity of the causative compounds?

The fact that large fish are more affected than small fish is not consistent with populations being exposed to the main 'culprits' - E_2, estrone and 17α-ethinylestradiol (Sumpter & Johnson, 2005). Furthermore, these compounds are relatively unstable (Johnson & Sumpter, 2001) and likely to be found in the coastal marine environment only in the neighbourhood of sewage treatment works and run-off water (Atkinson, Atkinson, & Tarrant, 2003). The causative agents are more likely to be persistent, probably relatively weak, estrogenic compounds. Many likely candidates have been identified by laboratory testing (Sumpter & Johnson, 2005), so it would be futile to approach this problem by trying to second-guess what the compounds might be. A better approach is to attempt to extract them from the tissues, bile or fat of affected fish, let them signal their presence with an *in vitro* estrogen screen, and then purify and identify them. This approach has been used successfully with freshwater fish

(Legler, Jonas, Lahr, Vethaak, Brouwer, & Murk, 2002; Gibson, Tyler, & Hill, 2005).

4.4. The problem of the 'outliers'.

The two male dabs with VTG concentrations above 10 µg/ml were reassayed to confirm that no mistake had been made when the plasma samples were assayed. The gonad sections were rechecked to make sure that the sex had been correctly ascribed. The plasma with the higher value is particularly extreme and difficult to explain. It is not without the bounds of possibility that, for this particular sample, a mistake was made in tube labelling or pipetting on board ship (since 1100 samples were collected). In favour of this being a case of mistaken identity is that liver sections of this fish could not be convincingly immunostained with the dab LV antibody (unpublished data). However, immunohistology is highly insensitive compared to ELISA.

We also examined our data to determine whether there was any possibility that any of the male samples might have been accidentally contaminated by an immediately preceding female sample (e.g. through accidental re-use of a syringe). However, we found no evidence of this (data not shown).

4.5. What can be considered a 'basal' VTG concentration?

Despite the lack of difference in VTG concentrations between the Irish Sea and the North Sea (after correcting for fish size) there were significant differences between individual sites and between clusters of sites. Such differences suggest that the fish either experience a different degree of exposure to environmental estrogens depending on where they live or that they belong to populations with different basal concentrations of VTG. In relation to the latter possibility, mean VTG concentrations from all sites plotted in rank order form a smooth distribution with no clear breaks, suggesting a single underlying continuous variable and arguing against there being 'separate populations'. Also, the five sites with the lowest mean VTG concentrations (< 0.1 µg/ml) were scattered between the three sea areas (three in the Irish Sea, one in the English Channel and one in the North Sea), suggesting that the 'basal

concentration' is the same (and at some value less than 0.1 μg/ml) wherever the fish come from.

It is impossible to define the basal concentration exactly (i.e. the concentration below which a male could be assumed not to have been exposed to environmental estrogens) because everywhere on earth is now affected to some extent by pollution. Hence, it is impossible to determine what is 'normal' (Sumpter and Johnson, 2005). We can only draw the conclusion that some dabs have been exposed to exogenous estrogens (albeit with very low levels of induction compared to cod and flounder) on the basis of relative differences between the sites and the fact that VTG concentrations are linked to fish size.

Other possible explanations for differences in VTG concentrations between sites include: the fish are all exposed to estrogens but fish at some sites respond more sensitively; the fish are all exposed to estrogens but that fish at some sites are also exposed to anti-estrogens; or that fish at some sites have more natural phytoestrogens in their diet than at others. The first two possibilities have recently been examined experimentally in the flounder and found to have little or no effect on VTG concentrations (Kirby et al., 2006; Kirby, Smith, Neall, Rooke, Scott, & Katsiadaki, in press). The third possibility cannot be easily dismissed. Our only argument against it is that, if phytoestrogens were the causative agents, it is unlikely that they would be cumulative or persistent and also unlikely that they would only be available to the larger fish.

4.6. Relationship of VTG concentrations to hepatosomatic index in males

The finding that HSI was positively correlated to VTG concentrations has been noted before in male flounders from the estuary of the River Mersey (Kleinkauf, Connor, Swarbreck, Levene, Walker, Johnson, & Leah, 2004a). One would fully expect to find this relationship in females, as the liver is the organ where the VTG is made and it would be expected to increase in weight as it expands protein production and takes on board the necessary precursors. However, no such correlation was found in the female dabs in the present study and since plasma VTG concentrations in male dabs (with the exception of the high outlier) were well below those in many of the females – and in fact all fell within the bottom 0.1 percentile of their full possible

range (i.e. the rate of production of VTG in even the most stimulated fish was likely to have been miniscule) - this seems an unlikely explanation. Further examination of our data revealed a correlation between HSI and fish weight ($r = 0.22$, $n = 357$, $p<0.0001$ for the males and $r = 0.37$, $n= 264$, $p<0.0001$ for females), suggesting that the relationship between VTG and HSI in males is probably just an 'association' (i.e. there is no direct causal link), although this does not mean that the increase in HSI with increase in fish size might not also be the result of the accumulation of persistent pollutants through the diet. However, it should be noted that, in the above-quoted study, HSI and body length were positively correlated in male flounders not only from the estuary of the River Mersey (heavy industrial contamination) but also from the River Dee (very low industrial contamination).

We have no good explanation for why, at three of the sites, mean HSI values were markedly higher than those found at the other sites - other than it might have reflected food availability. This was not assessed, however.

4.7. Evidence against the hypothesis that the presence of VTG in males is a natural ageing phenomenon.

The question most often asked at conferences where the results of the cod work (Scott et al., 2006b) have been presented is 'Could the appearance of VTG in the blood of large male fish be just a natural ageing phenomenon?'. One of the arguments against this from the cod study was that the fact that many of the affected male cod that were caught in the North Sea were actually quite young (only five years at the most) and were younger than completely unaffected male cod (six years old) caught off the Norwegian coast. The crucial difference was that the North Sea cod were much larger than the Norwegian cod. In the present study as well, the evidence points entirely to size and not age as the cause of raised VTG concentrations in dabs. The significant site differences in mean VTG concentrations (as discussed above) also suggest that the appearance of VTG in male dabs is not something that happens as part of an endogenous process. There were highly significant site differences in male swordfish, with no affected fish being found in the Pacific Ocean (Desantis et al., 2005). Similarly, male tuna caught in the Mediterranean had VTG in their plasma (Fossi et al., 2002) while tuna (*Thunnus obesus*) caught in the Pacific Ocean

(Hashimoto, Kurihara, Strussmann, Yamasaki, Soyano, Hara et al., 2003) did not. This dependence on where the fish are living reinforces the argument against the hypothesis that the appearance of VTG is something that happens as a matter of course to male fish as they grow older. Yet one further fact that can be used against this hypothesis is that there was a strong correlation between VTG induction and CYP1A induction (an enzyme involved in the deactivation of planar xenobiotics) in male swordfish, such that CYP1A was only present in affected males from the Atlantic Ocean and Mediterranean and absent from the unaffected males in the Pacific Ocean (Desantis et al., 2005). This finding is the strongest evidence yet that elevated VTG concentrations in male fish can be directly attributed to pollution.

Although fish could hypothetically make VTG without any stimulation, such a mechanism has not yet been demonstrated and in all studies to date, some form of hormonal induction, operating through a receptor, is required. The main natural endogenous signal in fish is E_2. Although E_2 could be measured in male cod, its levels were no different from those in immature females and were not correlated with VTG concentrations (Scott et al., 2006b). In the dab, E_2 was undetectable in most of the males that were examined.

4.8. VTG in females

Examination of ovaries, plus the results of the VTG assays, indicated that a high proportion of females (especially in the North Sea) had either not ended their previous reproductive cycle or were just beginning the next one. This concurs with observations (Htun-Han, 1978a, 1978b) that the reproductive cycle of the dab tends to extend throughout the year (i.e. there is possibly never a time when all individuals in the population are reproductively quiescent). In this situation, we feel it would be impossible to decide whether the presence of VTG in females, even those that appear to be immature (i.e. primary oocytes only), has an endogenous or exogenous cause. The reason for measuring VTG in female plasmas in future studies would be, as in the present study, to validate the assay. VTG differences in females were consistent with its role as the precursor of the egg yolk protein - being low in immature females and much higher in those showing signs of VTG incorporation or atresia in their ovaries. VTG was also highly correlated with maturity index and GSI in females. As our

females were more mature in the North Sea than in the Irish Sea, VTG concentrations were also significantly higher in the former area.

4.9. Would the dab be useful as a monitor of biological effects of estrogens?

In 2006, a report was submitted to the Working Group on Concentrations, Trends and Effects of Substances in the Marine Environment (Robinson & Scott, 2006) for consideration in relation to the OSPAR Convention for the Protection of the Marine Environment of the North East Atlantic. The main recommendations of the report were that: 1) in order to assess the occurrence, distribution, bioavailability and effects of estrogenic endocrine-disrupting chemicals in the OSPAR region, it is recommended that the determination of VTG in the blood plasma of male fish be added to the list of Contaminant-specific Biological Effects included in the OSPAR CEMP; 2) the recommended sentinel species are flounder (estuaries) and cod (offshore), which should be sampled in January/February. The committee reported back and accepted the first recommendation - plus the use of the flounder for monitoring estuaries. However, doubt was expressed about the choice of cod for offshore monitoring, because of its relative scarcity in some areas (especially of large male fish). The dab may be a better choice - with the advantage of demonstrated differences between sites. However, dab is lower in the food chain than adult cod, which, because the biomagnification factor of contaminants (and thus relative induction of VTG) is much lower, is a major disadvantage. Despite the successful outcome of the present study, if the dab were chosen as the sentinel species, it would be necessary to develop a more sensitive assay for VTG. Too many samples in the present study came close to or below the limits of the assay. This would (we suggest) be unacceptable for long-term trend data. The development of a more sensitive type of assay is not an insuperable problem (Fukada, Haga, Fujita, Hiramatsu, Sullivan, & Hara, 2001). It does require, however, considerably more effort and a higher degree of sophistication to develop than the type of ELISA described in this study.

4.10. What are the implications of estrogen exposure for the health of the fish?

This is the big question. As with freshwater fish, it is difficult to answer by studying fish in the field as there many other factors influence the survival of fish

populations, including fishing pressure and long-term climate change (Rose, 2004). The occurrence of males with intersex gonads (Lye et al., 1997; Allen et al., 1999b; Cho, Kurihara, Strussmann, Uozumi, Yamakawa, Yamasaki et al., 2003; De Metrio et al., 2003) or feminised secondary sexual characteristics (Kirby, Bignell, Brown, Craft, Davies, Dyer et al., 2003) has been directly ascribed to estrogenic exposure. Although, two intersex dab were found in the Dogger Bank area during the CSEMP survey in 2003 (Stentiford & Feist, 2005), none was found in the 2004 and 2005 surveys. This rarity of intersex in dab could be taken as evidence that the small amount of estrogen to which they are apparently being exposed is not detrimental to the health of individuals or populations. However, intersex is neither a very specific nor very sensitive outcome of estrogen exposure in most species.

As already mentioned, VTG concentrations in male dabs lie within the bottom 0.1 percentile of possible VTG concentrations (assuming a VTG concentration of 10 mg/ml in a fully mature female or an E_2-treated male). In cod, values lie within the bottom 2.0 percentile and, in flounder, occupy, and in some cases exceed, the entire range. On the basis of such differences, it would be tempting to conclude that the degree of estrogen exposure to which the dabs are being exposed is likely to be of little or no biological significance. However, with our present state of knowledge, it is dangerous to make such judgements. If, as we hypothesise, the causative agents in dab and cod are cumulative and persistent, VTG induction might just be the 'tip of the iceberg' in terms of biological and, ultimately, population effects.

We believe that we will only make headway in answering the question of population effects if we know what sorts of compounds (and routes of exposure) we are dealing with in marine fish. This information is also needed by governments to make rational decisions on how to deal with any problems (e.g. possible concentration further up the food chain to mammals that eat fish). Such an approach would also help to resolve whether the changes that are observed in cod and dab might have a simple natural cause such as ingestion of naturally-occurring phytoestrogens.

Acknowledgements

This work was primarily funded by the Chemicals and Nanotechnologies Division, Department for the Environment, Food and Rural Affairs (Defra), UK (contract

CB01706). Dab collection and histology (under the auspices of CSEMP) were funded by Marine and Environmental Division, Defra, UK (contract AE003). We thank the numerous CEFAS staff who were involved in the collection of the plasma samples, histology, fish ageing and statistical analysis.

Figure captions

Fig. 1. UV absorption trace following anion-exchange chromatography of crude precipitated dab lipovitellin. The thinner line shows the gradient of 1M NaCl that was applied to the column: from 5% at the beginning to 100% at the top. Only the first major peak (A: 90-130 ml) cross-reacted with the antiserum to dab lipovitellin.

Fig. 2. Cross-reaction in the ELISA of five-fold dilutions of purified dab lipovitellin (solid circles), purified dab vitellogenin (solid squares) and four randomly chosen female plasma samples (open symbols) that had been diluted 1:10 before being added to the wells. In two separate experiments, there was a high correlation in concentrations of vitellogenin in samples that had been measured using either lipovitellin or vitellogenin as plate-coating material and standard (r^2 = 0.87, n=60 in first experiment and 0.90, n=40 in second experiment, p<0.0001 for both).

Fig. 3. Map of mean plasma vitellogenin concentrations (µg/ml) in male dab. The heights of the bars indicates the mean vitellogenin concentration. The highest bar represents a value of 1.6 µg/ml. Data taken from Table 2. Two outlying observations are excluded (Table 3).

Fig. 4. Vitellogenin concentrations (µg/ml) v. fish weight (plus least-squares regression line) for all but two (outlier) male dabs caught in June/July 2005.

Fig. 5. Vitellogenin concentrations (µg/ml) v. fish age for males that were caught in the Dogger Bank area of the North Sea (sites 18 to 21). Relationship not statistically significant.

Fig. 6. Vitellogenin concentrations (µg/ml) v. weight (plus least-squares regression line) for males that were caught in the Dogger Bank area of the North Sea (sites 18 to 21). Same males as shown in Fig. 5.

Fig. 7. Mean ± SEM concentration of 17β-estradiol (lower graph) in female dab v. stage of maturation of the ovaries and also based upon whether they had 'high' or 'low' vitellogenin concentrations (upper graph). Each group had ten fish, except the first group that had an outlier (primary stage of development; plasma vitellogenin = 5 µg/ml; E_2 > 20 ng/ml) that was excluded from the analysis. The only group in which E_2 concentrations were significantly different from the other groups is marked with an asterisk. The stages of development were

based upon the most advanced stage of oocytes present in the ovaries: primary; cortical alveoli; vitellogenic (essentially the new season's growing oocytes) and atretic (essentially the previous season's unused oocytes).

Table 1 Location of sites of capture of dab in June/July 2005

Site No.	Date of capture	Site name	Latitude	Longitude	Depth (m)
1	June 17th, 2005	Carmarthan Bay	51.55N	4.65W	40
2	June 18th, 2005	Inner Cardigan Bay	52.18N	4.51W	25
3	June 18th, 2005	South Cardigan Bay	52.31N	4.26W	24
4	June 19th, 2005	North Cardigan Bay	52.70N	4.54W	51
5	June 20th, 2005	Dundrum Bay	54.08N	5.62W	46
6	June 21st, 2005	St Bee's	54.55N	3.85W	29
7	June 21st, 2005	SE Isle of Man	54.06N	3.82W	47
8	June 22nd, 2005	Morecambe Bay	53.90N	3.40W	18
9	June 23rd, 2005	Burbo Bight	53.47N	3.32W	19
10	June 24th, 2005	Liverpool Bay	53.47N	3.70W	34
11	June 24th, 2005	Red Wharf Bay	53.37N	4.17W	44
12	June 25th, 2005	West Lundy	51.19N	5.46W	81
13	June 28th, 2005	Lyme Bay	50.61N	2.91W	31
14	June 28th, 2005	Eddystone	50.11N	4.15W	73
15	June 29th, 2005	Newhaven	50.76N	0.11W	21
16	June 30th, 2005	Rye Bay	50.76N	0.75E	39
17	June 30th, 2005	Outer Gabbard	52.04N	2.11E	45
18	July 2nd, 2005	Central Dogger	54.52N	2.69E	24
19	July 2nd, 2005	NE Dogger	55.27N	2.90E	32
20	July 3rd, 2005	West Dogger	54.79N	1.28E	37
21	July 3rd, 2005	North Dogger	55.06N	2.06E	32
22	July 4th, 2005	Off Flamborough	54.25N	0.48E	64
23	July 4th, 2005	Off Humber	54.08N	1.81E	82
24	July 7th, 2005	Indefatigable Bank	53.57N	2.09E	51
25	July 7th, 2005	Amble	55.25N	1.26W	68
26	July 8th, 2005	Tees Bay	54.77N	1.14W	37

Table 2 Summary statistics of male dab (Mean ± S.E.)

Site No.	Site	n	Length (cm)	Weight (g)	HSI	VTG (µg/ml)	Maturity Index
1	Carmarthan Bay	15	21.9 ± 0.6	111.7 ± 9.6	2.0 ± 0.2	0.828 ± 0.383	1.0
2	Inner Cardigan Bay	12	17.8 ± 0.3	51.2 ± 3.2	1.3 ± 0.1	0.044 ± 0.014	1.1
3	South Cardigan Bay	10	18.0 ± 0.4	52.7 ± 2.8	1.1 ± 0.1	0.062 ± 0.019	1.0
4	North Cardigan Bay	9	16.9 ± 0.7	45.1 ± 5.4	1.4 ± 0.1	0.086 ± 0.022	1.0
5	Dundrum Bay	6	17.3 ± 0.5	55.5 ± 4.0	1.7 ± 0.1	0.329 ± 0.178	1.3
6	St Bee's	20	18.7 ± 0.3	66.6 ± 3.6	1.4 ± 0.1	0.137 ± 0.021	1.8
7	SE Isle of Man	35	20.4 ± 0.2	88.0 ± 3.1	1.4 ± 0.0	0.266 ± 0.091	1.0
8	Morecambe Bay	15	19.9 ± 0.4	80.2 ± 5.1	1.6 ± 0.1	0.196 ± 0.057	1.0
9	Burbo Bight	23	18.8 ± 0.2	64.2 ± 2.1	1.7 ± 0.1	0.501 ± 0.356	1.8
10	Liverpool Bay	21	20.7 ± 0.5	93.1 ± 5.9	1.8 ± 0.1	0.583 ± 0.259	2.0
11	Red Wharf Bay	15	19.3 ± 0.4	84.0 ± 6.3	2.4 ± 0.3	0.407 ± 0.129	1.9
12	West Lundy	10	20.3 ± 0.5	87.9 ± 6.4	1.8 ± 0.1	0.232 ± 0.066	1.9
13	Lyme Bay	20	19.8 ± 0.3	93.1 ± 4.6	2.6 ± 0.1	0.193 ± 0.040	2.0
14	Eddystone	21	19.9 ± 0.3	94.8 ± 3.8	2.6 ± 0.1	0.565 ± 0.178	1.9
15	Newhaven	16	20.2 ± 0.6	87.8 ± 7.2	1.5 ± 0.1	0.118 ± 0.021	2.0
16	Rye Bay	18	20.2 ± 0.4	84.2 ± 5.6	1.8 ± 0.3	0.069 ± 0.017	1.9
17	Outer Gabbard	10	22.5 ± 0.5	115.2 ± 6.7	1.5 ± 0.1	0.150 ± 0.058	1.8
18	Central Dogger	22	20.6 ± 0.2	86.5 ± 2.9	1.7 ± 0.1	0.378 ± 0.143	1.7
19	NE Dogger	24	23.0 ± 0.3	122.0 ± 5.3	1.2 ± 0.1	1.600 ± 0.450	1.6
20	West Dogger	22	20.9 ± 0.3	87.9 ± 4.7	1.3 ± 0.1	0.277 ± 0.052	1.4
21	North Dogger	22	21.3 ± 0.4	98.4 ± 5.3	1.6 ± 0.1	0.377 ± 0.154	1.8
22	Off Flamborough	23	20.2 ± 0.2	79.8 ± 2.9	1.9 ± 0.1	1.353 ± 0.934	1.7
23	Off Humber	16	20.9 ± 0.4	91.4 ± 4.3	1.8 ± 0.1	0.548 ± 0.120	2.0
24	Indefatigable Bank	11	20.5 ± 0.4	90.0 ± 6.0	1.7 ± 0.1	0.172 ± 0.091	2.0
25	Amble	17	21.0 ± 0.3	89.4 ± 4.6	1.8 ± 0.1	0.678 ± 0.231	1.8
26	Tees Bay	15	22.3 ± 0.3	114.1 ± 4.2	1.8 ± 0.1	0.067 ± 0.012	1.7

Two outliers were excluded (data in Table 3)

Table 3 Sites and date of capture of two male dab outliers (high VTG).

Site No.	Date of capture	Site name	Length (cm)	Weight (g)	Liver weight (g)	E_2 (ng/ml)	VTG (µg/ml)	Maturity index
22	July 4th, 2005	Off Flamborough	21	84	1.3	<0.04	21.6	1
10	June 24th, 2005	Liverpool Bay	22	116	3.9	<0.04	755.8	2

Table 4 Summary statistics of female dab (Mean ± S.E.)

Site No.	Date of capture	Site name	n	Length (cm)	Weight (g)	HSI	GSI	VTG (µg/ml)	Maturity index
1	June 17th, 2005	Carmarthan Bay	12	24.3 ± 0.7	161.3 ± 14.6	2.3 ± 0.3	0.8 ±0.1	673.2 ± 229.6	1.0
2	June 18th, 2005	Inner Cardigan Bay	10	21.3 ± 0.4	89.5 ± 7.0	1.5 ± 0.1	0.8 ± 0.1	107.6 ± 30.2	1.0
3	June 18th, 2005	South Cardigan Bay	10	19.0 ± 0.5	62.8 ± 3.4	1.1 ± 0.0	0.8 ± 0.1	225.2 ± 77.7	1.0
4	June 19th, 2005	North Cardigan Bay	10	22.2 ± 0.3	97.2 ± 3.2	1.5 ± 0.2	0.8 ± 0.1	84.9 ± 62.0	1.0
5	June 20th, 2005	Dundrum Bay	5	18.4 ± 0.9	63.0 ± 9.6	1.5 ± 0.2	0.7 ± 0.1	117.1 ± 76.4	1.0
6	June 21st, 2005	St Bee's	10	22.0 ± 0.8	114.8 ± 10.0	1.5 ± 0.2	0.9± 0.1	771.4 ± 216.3	1.3
7	June 21st, 2005	SE Isle of Man	10	23.3 ± 0.9	129.0 ± 17.0	1.5 ± 0.2	0.9 ± 0.1	574.6 ± 267.8	1.3
8	June 22nd, 2005	Morecambe Bay	10	23.7 ± 0.6	145.6 ± 12.1	1.8 ± 0.3	0.9 ± 0.1	562.2 ± 106.2	1.2
9	June 23rd, 2005	Burbo Bight	10	21.8 ± 0.7	105.7 ± 9.5	2.0 ± 0.2	0.8 ± 0.1	457.2 ± 203.2	1.1
10	June 24th, 2005	Liverpool Bay	10	25.6 ± 0.7	168.6 ± 14.5	1.8 ± 0.2	1.1 ± 0.3	584.5 ± 244.4	1.4
11	June 24th, 2005	Red Wharf Bay	10	23.7 ± 1.1	158.7 ± 24.5	2.2 ± 0.2	0.9 ± 0.1	146.5 ± 69.9	1.7
12	June 25th, 2005	West Lundy	10	20.2 ± 0.2	83.1 ± 3.3	1.4 ± 0.0	0.6 ± 0.0	242.1 ± 91.3	1.8
13	June 28th, 2005	Lyme Bay	20	22.6 ± 0.9	149.2 ± 20.8	2.4 ± 0.1	0.5 ± 0.0	126.1 ± 68.4	2.2
14	June 28th, 2005	Eddystone	9	19.9 ± 0.4	87.8 ± 4.7	2.4 ± 0.2	0.5 ± 0.0	94.1 ± 53.3	2.1
15	June 29th, 2005	Newhaven	10	22.2 ± 0.6	124.4 ± 12.0	1.8 ± 0.2	0.6 ± 0.0	214.5 ± 121.5	1.6
16	June 30th, 2005	Rye Bay	9	23.1 ± 0.5	121.8 ± 9.0	1.2 ± 0.2	1.1 ± 0.0	348.2 ± 152.7	1.8
17	June 30th, 2005	Outer Gabbard	10	23.2 ± 0.4	127.8 ± 8.1	1.3 ± 0.1	0.8 ± 0.1	1369.7 ± 599.9	1.9
18	July 2nd, 2005	Central Dogger	10	24.2 ± 0.6	143.1 ± 11.2	1.4 ± 0.1	1.2 ± 0.1	2776.2 ± 745.3	2.6
19	July 2nd, 2005	NE Dogger	11	24.1 ± 0.4	130.7 ± 7.9	2.2 ± 0.1	1.9 ± 0.6	1675.8 ± 561.6	2.6
20	July 3rd, 2005	West Dogger	10	21.7 ± 0.5	105.7 ± 8.1	1.3 ± 0.1	1.2 ± 0.2	2537.4 ± 541.9	2.9
21	July 3rd, 2005	North Dogger	10	23.2 ± 0.7	130.1 ± 9.0	1.3 ± 0.1	2.0 ± 0.5	3936.9 ± 924.5	3.1
22	July 4th, 2005	Off Flamborough	11	23.5 ± 0.5	121.5 ± 8.0	1.3 ± 0.1	1.1 ± 0.1	3185.9 ± 1138.9	3.0
23	July 4th, 2005	Off Humber	10	23.3 ± 0.6	123.6 ± 9.4	1.5 ± 0.1	1.3 ± 0.1	2341.6 ± 456.0	3.2
24	July 7th, 2005	Indefatigable Bank	10	24.3 ± 0.7	144.0 ± 11.6	1.9 ± 0.1	0.9 ± 0.1	765.7 ± 282.0	2.3
25	July 7th, 2005	Amble	10	25.3 ± 0.7	167.8 ± 15.5	-	-	1653.2 ± 515.9	2.5
26	July 8th, 2005	Tees Bay	11	22.5 ± 0.5	120.5 ± 6.7	1.8 ± 0.1	1.0 ± 0.1	890.2 ± 208.8	2.7

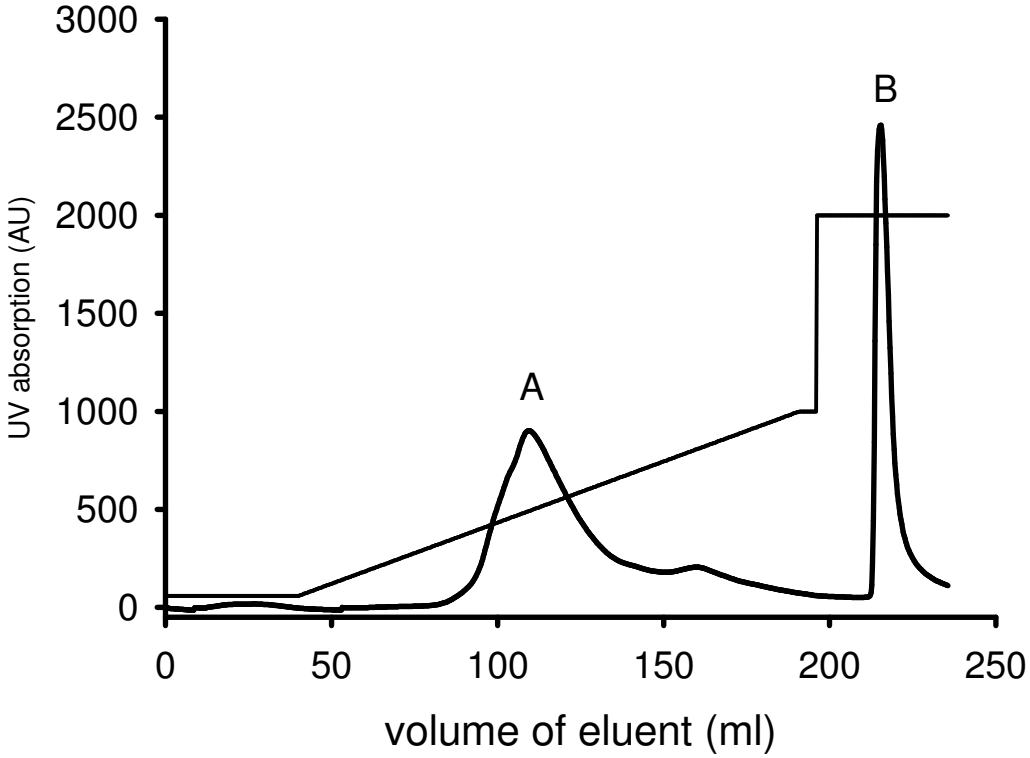


Fig 1 above

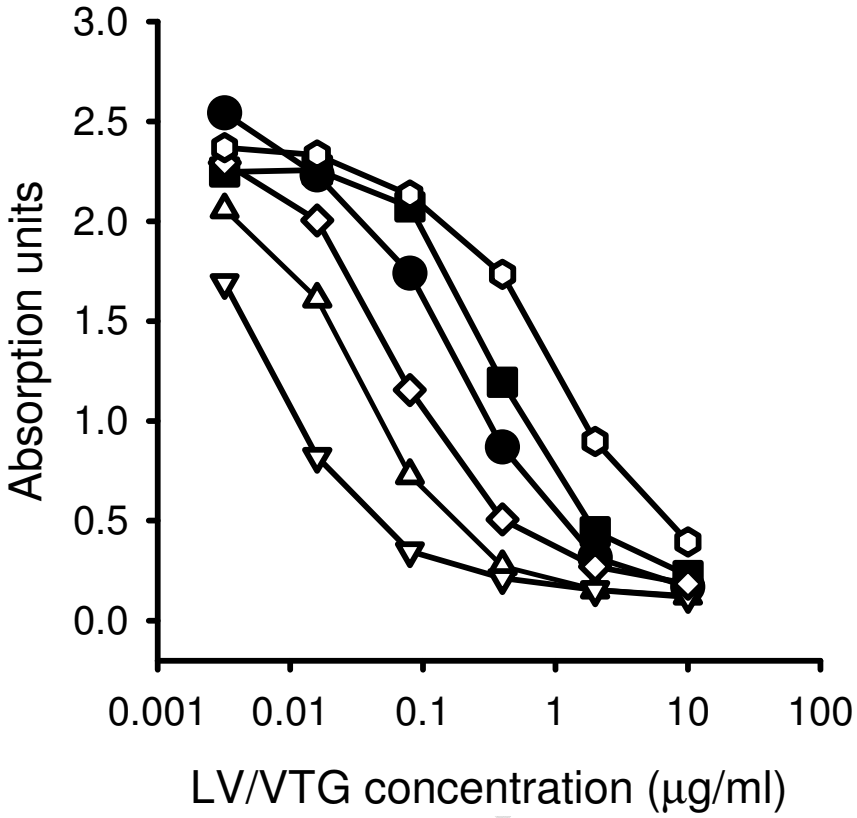


Fig 2 above

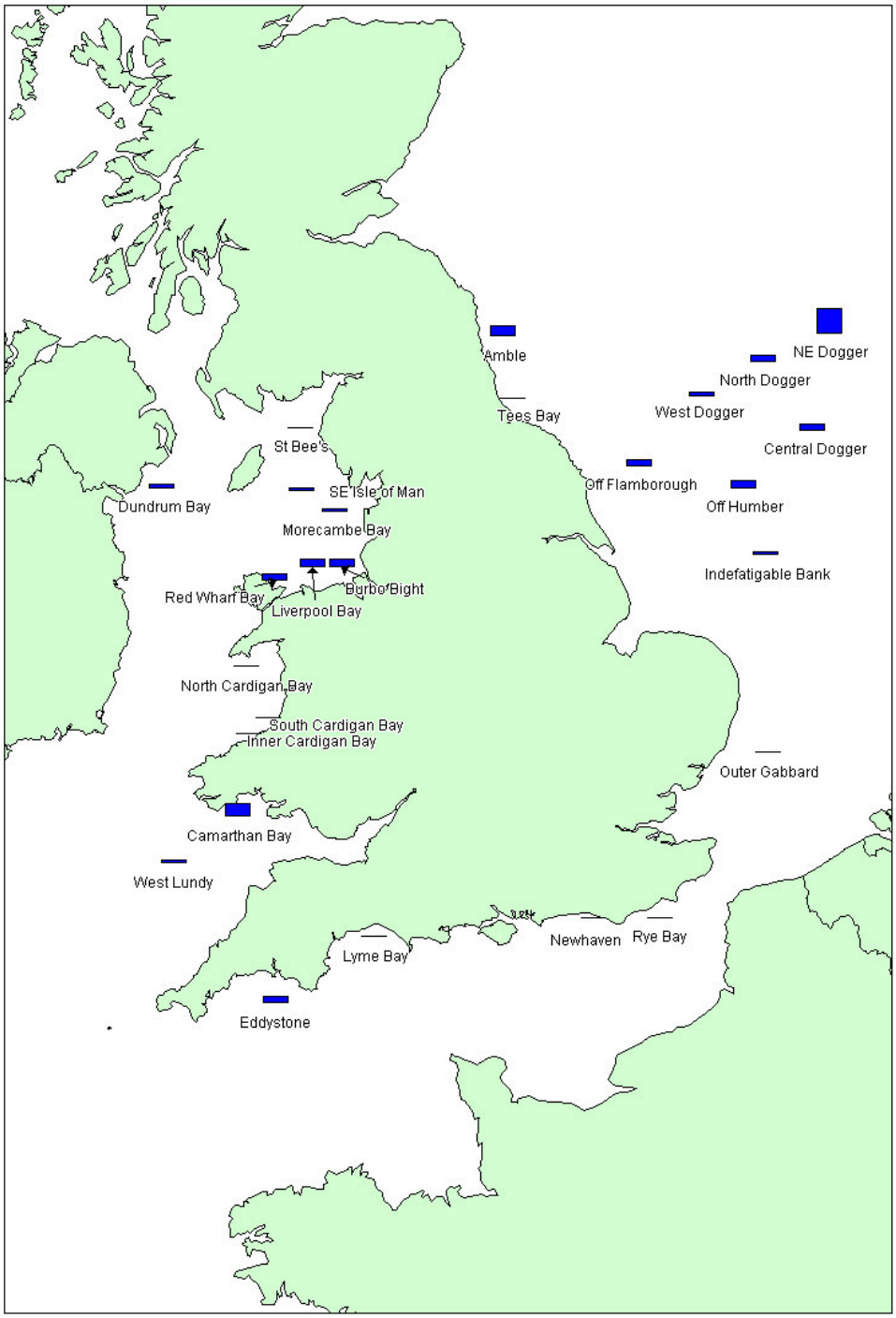

Amble
NE Dogger
North Dogger
West Dogger
Tees Bay
Central Dogger
St Bee's
Off Flamborough
Off Humber
Dundrum Bay
SE Isle of Man
Morecambe Bay
Indefatigable Bank
Red Wharf Bay
Burbo Bight
Liverpool Bay
North Cardigan Bay
South Cardigan Bay
Inner Cardigan Bay
Outer Gabbard
Camarthan Bay
West Lundy
Newhaven
Rye Bay
Lyme Bay
Eddystone

Fig 3 above

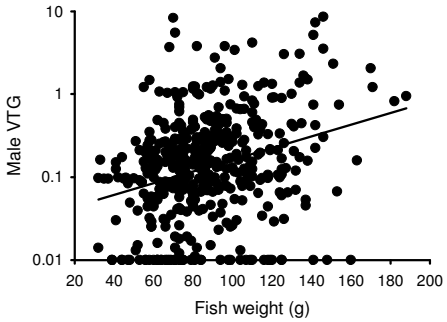


Fig 4 above

Fig 5 above

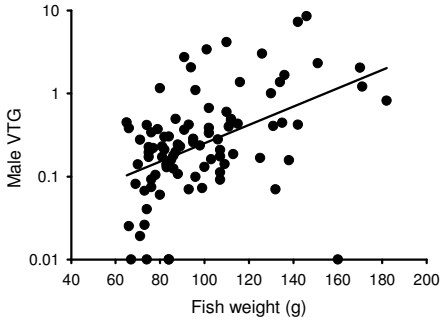


Fig 6 above

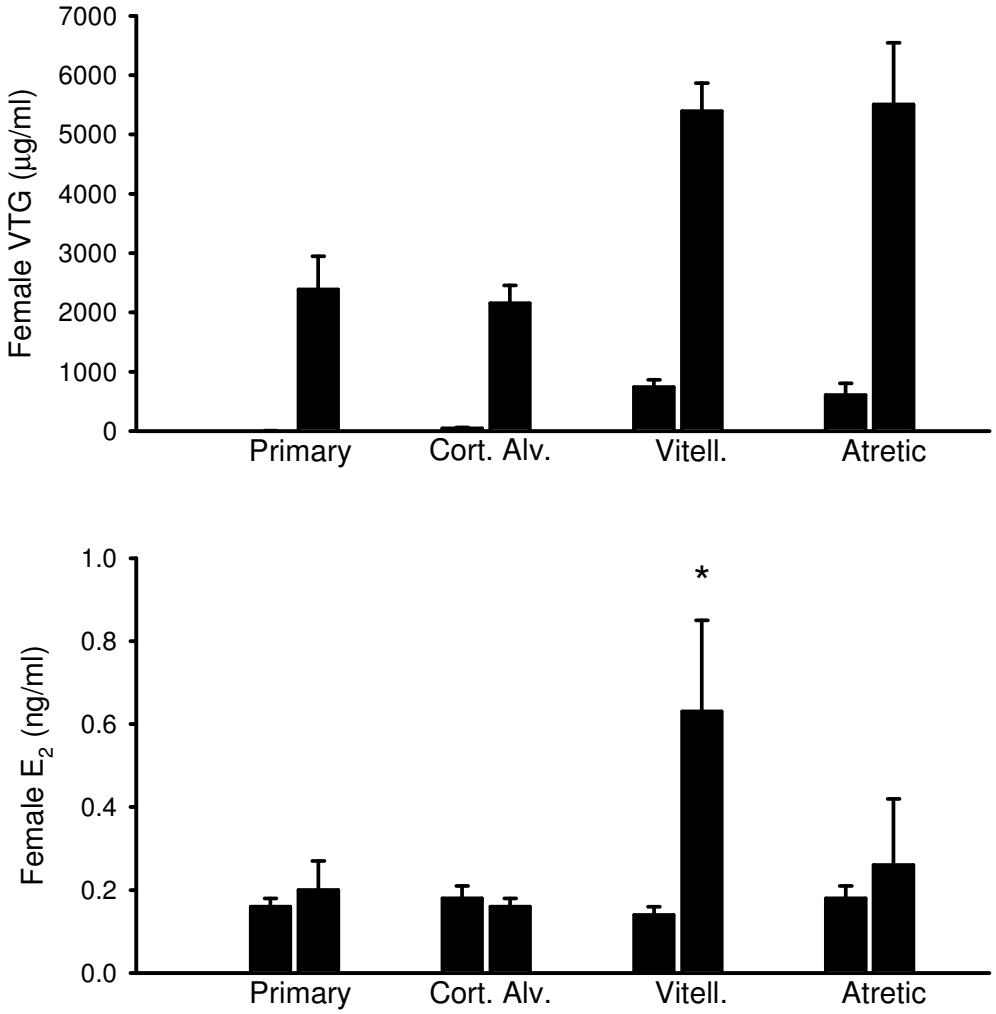

7000
6000
5000
4000
3000
2000
1000
0
Female VTG (µg/ml)
Primary
Cort. Alv.
Vitell.
Atretic
1.0
0.8
0.6
0.4
0.2
0.0
Female E2 (ng/ml)
*
Primary
Cort. Alv.
Vitell.
Atretic

Fig 7 above

Allen, Y., Matthiessen, P., Scott, A. P., Haworth, S., Feist, S., & Thain, J. E. (1999a). The extent of oestrogenic contamination in the UK estuarine and marine environments - further surveys of flounder. *Science of the Total Environment, 233*, 5-20.

Allen, Y., Scott, A. P., Matthiessen, P., Haworth, S., Thain, J. E., & Feist, S. (1999b). Survey of estrogenic activity in United Kingdom estuarine and coastal waters and its effects on gonadal development of the flounder *Platichthys flesus*. *Environmental Toxicology and Chemistry, 18*, 1791-1800.

Atkinson, S., Atkinson, M. J., & Tarrant, A. M. (2003). Estrogens from sewage in coastal marine enviornments. *Environmental Health Perspectives, 111*, 531-535.

Barucca, M., Canapa, A., Olmo, E., & Regoli, F. (2006). Analysis of vitellogenin gene induction as a valuable biomarker of estrogenic exposure in various Mediterannean fish species. *Environmental Research, 101*, 68-73.

Burreau, S., Zebuhr, Y., Broman, D., & Ishaq, R. (2006). Biomagnification of PBDEs and PCBs in food webs from the Baltic Sea and the northern Atlantic Ocean. *Science of the Total Environment, 366*, 659-672.

Cho, S.-M., Kurihara, R., Strussmann, C. A., Uozumi, M., Yamakawa, H., Yamasaki, T., et al. (2003). Histological abnormalities in the gonads of konoshiro gizzard shad (*Konosirus punctatus*) from coastal areas of Japan. *Environmental Sciences, 10*, 25-36.

Daan, N. (1973). A quantitative analysis of the food intake of North Sea cod, *Gadus morhua*. *Netherlands Journal of Sea Research, 6*, 479-517.

De Metrio, G., Corriero, A., Desantis, S., Zubani, D., Cirillo, F., Deflorio, M., et al. (2003). Evidence of a high percentage of intersex in the Mediterranean swordfish (*Xiphias gladius* L.). *Marine Pollution Bulletin, 46*, 358-361.

Desantis, S., Corriero, A., Cirillo, F., Deflorio, M., Brill, R., Griffiths, M., et al. (2005). Immunohistochemical localization of CYP1A, vitellogenin and zona radiata proteins in the liver of swordfish (*Xiphias gladius* L.) taken from the Mediterranean Sea, South Atlantic, South Western Indian and Central North Pacific Oceans. *Aquatic Toxicology, 71*, 1-12.

Fossi, M. C., Casini, S., Ancora, S., Moscatelli, A., Ausili, A., & Notarbartolo-di-Sciara, G. (2001). Do endocrine disrupting chemicals threaten Mediterranean swordfish? Preliminary results of vitellogenin and *zona radiata* proteins in *Xiphias gladius*. *Marine Environmental Research, 52*, 477-483.

Fossi, M. C., Casini, S., Marsili, L., Ancora, S., Mori, G., Neri, G., et al. (2004). Evaluation of ecotoxicological effects of endocrine disrupters during a four-year survey of the Mediterranean population of swordfish (*Xiphias gladius*). *Marine Environmental Research, 58*, 425-429.

Fossi, M. C., Casini, S., Marsili, L., Neri, G., Mori, G., Ancora, S., et al. (2002). Biomarkers for endocrine disruption in three species of Mediterranean large pelagic fish. *Marine Environmental Research, 54*, 667-671.

Fukada, H., Haga, A., Fujita, T., Hiramatsu, N., Sullivan, C. V., & Hara, A. (2001). Development and validation of chemiluminescent immunoassay for vitellogenin in five salmonid species. *Comparative Biochemistry and Physiology, 130A*, 163-170.

Gibson, R., Tyler, C. R., & Hill, E. M. (2005). Analytical methodology for the identification of estrogenic contaminants in fish bile. *Journal of Chromatography A, 1066*, 33-40.

Hara, A., Matsubara, H., & Soyano, K. (2001). Endocrine and sexual disruption in wild grey mullet. In Japan, O. E. C. C. (Ed.), *UK-Japan Research Cooperation on Endocrine Disrupting Chemicals* (pp. 42-46). Tokyo: Ministry of the Environment of Japan.

Hashimoto, S., Bessho, H., Hara, A., Nakamura, M., Iguchi, T., & Fujita, K. (2000). Elevated serum vitellogenin levels and gonadal abnormalities in wild male flounder (*Pleuronectes yokohamae*) from Tokyo Bay, Japan. *Marine Environmental Research, 49*, 37-53.

Hashimoto, S., Kurihara, R., Strussmann, C. A., Yamasaki, T., Soyano, K., Hara, A., et al. (2003). Gonadal histology and serum vitellogenin levels of bigeye tuna *Thunnus obesus* from the northern Pacific ocean - absence of endocrine disruption bio-indicators. *Marine Pollution Bulletin, 46*, 459-465.

Htun-Han, M. (1978a). The reproductive biology of the dab *Limanda limanda* (L.) in the North Sea: seasonal changes in the testis. *Journal of Fish Biology, 13*, 361-367.

Htun-Han, M. (1978b). The reproductive biology of the dab Limanda limanda (L.) in the. North Sea: Seasonal changes in the ovary. *Journal of Fish Biology, 13*, 351-359.

Jobling, S., Williams, R, Johnson, A., Taylor, A., Gross-Sorokin, M., Nolan, M., et al. (2006). Predicted exposures to steroid estrogens in U.K. rivers correlate with

widespread sexual disruption in wild fish populations. *Environmental Health Perspectives,* 114 (Suppl. 1), 32-39.

Johnson, A. C., & Sumpter, J. P. (2001). Removal of endocrine-disrupting chemicals in activated sludge treatment works. *Environmental Science and Technology, 35*, 4697-4703.

Katsiadaki, I., Scott, A. P., Hurst, M. R., Matthiessen, P., & Mayer, I. (2002). Detection of environmental androgens: a novel method based on enzyme-linked immunosorbent assay of spiggin, the stickleback (*Gasterosteus aculeatus*) glue protein. *Environmental Toxicology and Chemistry, 21*, 1946-1954.

Kirby, M. F., Bignell, J., Brown, E., Craft, J. A., Davies, I., Dyer, R. A., et al. (2003). The presence of morphologically intermediate papilla syndrome in United Kingdom pupulations of sand goby (*Pomatoschistus* spp.): endocrine disruption. *Environmental Toxicology and Chemistry, 22*, 239-251.

Kirby, M. F., Allen, Y. T., Dyer, R. A., Feist, S. W., Katsiadaki, I., Matthiessen, P., et al. (2004). Surveys of plasma vitellogenin and intersex in male flounder (*Platichthys flesus*) as measures of endocrine disruption by estrogenic contamination in United Kingdom estuaries: temporal trends, 1996 to 2001. *Environmental Toxicology and Chemistry, 23*, 748-758.

Kirby, M. F., Smith, A. J., Barry, J., Katsiadaki, I. Lyons, B., & Scott, A. P. (2006). Differential sensitivity of flounder (*Platichthys flesus*) in response to oestrogenic chemical exposure: an issue for design and interpretation of monitoring and research programmes. *Marine Environmental Research,* 62, 315-325.

Kirby, M. F. Smith, A. J., Neall, P., Rooke, J., Scott, A. P., & Katsiadaki, I. Ethoxyresorufin-O-deethylase (EROD) and vitellogenin in flounder (*Platichthys flesus*): System interaction, crosstalk and implications for monitoring. *Aquatic Toxicology,* (in press)

Kleinkauf, A., Connor, L., Swarbreck, D., Levene, C., Walker, P., Johnson, P. J. & Leah (2004a). General condition biomarkers in relation to contaminat burden in European flounder (*Platicthys flesus*). *Ecotoxicology and Environmental Safety, 58*, 335-355.

Kleinkauf, A., Scott, A. P., Stewart, C., Simpson, M. G., & Leah, R. T. (2004b). Abnormally elevated VTG concentrations in flounder (*Platichthys flesus*) from

the Mersey Estuary (UK) - a continuing problem. *Ecotoxicology and Environmental Safety, 58*, 356-364.

Legler, J., Jonas, A., Lahr, J., Vethaak, A. D., Brouwer, A., & Murk, A. J. (2002). Biological measurement of estrogenic activity in urine and bile conjugates with the in vitro ER-Calux reporter gene assay. *Environmental Toxicology and Chemistry, 21*, 473-479.

Lye, C. M., Frid, C. L. J., & Gill, M. E. (1998). Seasonal reproductive health of flounder *Platicthys flesus* exposed to sewage effluent. *Marine Ecology Progress Series, 170*, 249-260.

Lye, C. M., Frid, C. L. J., Gill, M. E., & McCormick, D. (1997). Abnormalities in the reproductive health of flounder *Platichthys flesus* exposed to effluent from a sewage treatment works. *Marine Pollution Bulletin, 34*, 34-41.

Matthiessen, P., Allen, Y. T., Bamber, S., Craft, J., Hurst, M. R., Hutchinson, T. H., et al. (2002). The impact of oestrogenic and androgenic contamination on marine organisms in the United Kingdom - summary of the EDMAR programme. *Marine Environmental Research, 54*, 645-649.

Mills, L. J., Gutjahr-Gobell, R., Horowitz, D. B., Denslow, N., Chow, M. C., & Zaroogian, G. (2003). Relationship between reproductive success and male plasma vitellogenin concentrations in cunner, *Tautogolabrus adspersus*. *Environmental Health Perspectives, 111*, 93-99.

Ohkubo, N., Mochida, K., Adachi, S., Hara, A., Hotta, K., Nakamura, Y., et al. (2003). Estrogenic activity in coastal areas around Japan evaluated by measuring male serum vitellogenins in Japanese common goby *Acanthogobius flavimanus*. *Fisheries Science, 69*, 1135-1145.

Olsson, A., Valters, K., & Burreau, S. (2000). Concentrations of organochlorine substances in relation to fish size and trophic position: a study on perch (*Perca fluviatilis* L.). *Environmental Science and Technology, 34*, 4878-4886.

Plack, P. A., Pritchard, D. J., & Fraser, N. W. (1971). Egg proteins in cod serum. Natural occurrence and induction by injections of oestradiol 3-benzoate. *Biochemical Journal, 121*, 847-856.

Purdom, C. E., Hardiman, P. A., Bye, V. J., Eno, N. C., Tyler, C. R., & Sumpter, J. P. (1994). Estrogenic effects of effluents from sewage treatment works. *Chemistry and Ecology, 8*, 275-285.

Robinson, C. D., & Scott, A. P. (2006). Draft OSPAR Background Document for Biological Effects Monitoring Techniques: Fish Vitellogenin as a Biomarker of Exposure to Xenoestrogens. Fisheries Research Services Collaborative Report No. 09/06. Aberdeen: Fisheries Research Services, Marine Laboratory, Victoria Road, Aberdeen, AB11 9DB, UK, 29 pp.

Rose, G. A. (2004). Reconciling overfishing and climate change with stock dynamics of Atlantic cod (*Gadus morhua*) over 500 years. *Canadian Journal of Fisheries and Aquaculture Science, 61*, 1553-1557.

Roy, L. A., Armstrong, J. L., Sakamoto, K., Steinert, S., Perkins, E., Lomax, D. P., et al. (2003). The relationship of biochemical endpoints to histopathology and population metrics in feral flatfish species collected near the municipal wastewater outfall of Orange County, California, USA. *Environmental Toxicology and Chemistry, 22*, 1309-1317.

Scott, A. P., Stewart, C., Allen, Y., & Matthiessen, P. (2000). 17β-oestradiol in male flatfish. In: Norberg, B., Kjesbu, O. S., Taranger, G. L., Andersson, E. & Stefansson, S. O. (Eds.), *Proceedings of the Sixth International Symposium on Reproductive Physiology of Fish, Bergen, Norway, July 4-9, 1999.*, (pp. 382). Bergen, Norway: Bergen 2000.

Scott, A. P., Katsiadaki, I., Kirby, M., & Thain, J. (2006a). Relationship between sex steroid and vitellogenin concentrations in flounder (*Platichthys flesus*) sampled from an estuary contaminated with estrogenic endocrine-disrupting compounds. *Environmental Health Perspectives, 114 (Suppl. 1)*, 27-31.

Scott, A. P., Katsiadaki, I., Witthames, P. R., Hylland, K., Davies, I. M., McIntosh, A. D., et al. (2006b). Vitellogenin in the blood plasma of male cod (*Gadus morhua*): a sign of oestrogenic endocrine disruption in the open sea? *Marine Environmental Research, 61*, 149-170.

Stentiford, G. D., & Feist, S. W. (2005). First reported case of intersex (ovotestis) in the flatfish species dab *Limanda limanda*: Dogger Bank, North Sea. *Marine Ecology Progress Series, 301*, 307-310.

Sumpter, J. P., & Johnson, A. C. (2005). Lessons from endocrine disruption and their application to other issues concerning trace organics in the aquatic environment. *Environmental Science and Technology, 39*, 4321-4332.

Thomas, K. V., Balaam, J., Hurst, M. R., Nedyalkova, Z., & Mekenyan, O. (2004). Potency and characterization of estrogen-receptor agonists in United Kingdom estuarine sediments. *Environmental Toxicology and Chemistry, 23*, 471-479.

Vethaak, A. D., Lahr, J., Kuiper, R. V., Grinwis, G. C. M., Rankouhi, T. R., Giesy, J. P., et al. (2002). Estrogenic effects in fish in The Netherlands: some preliminary results. *Toxicology, 181-182*, 147-150.

Vine, E., Shears, J., van Aerle, R., Tyler, C. R., & Sumpter, J. P. (2005) Endocrine (sexual) disruption is not a prominent feature in the pike (*Esox lucius*), a top predator, living in English waters. *Environmental Toxicology and Chemistry* 114, 1436-1443.